US ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY

REPORT NO. 568

MEASUREMENT OF ADP:O VALUES IN RAT LIVER HOMOGENATES. II. EFFECTS OF PURINE AND PYRIMIDINE NUCLEOTIDES ON RESPIRATION AND PHOSPHORYLATION

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UNITED STATES ARMY
MEDICAL RESEARCH AND DEVELOPMENT COMMAND

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The animals used in this study were handled in accordance with the "Principles of Laboratory Animal Care" established by the National Society for Medical Research.

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1. Nucleotide Metabolism
2. Oxidative Phosphorylation

Report No. 568, 20 May 63, 21 pp & i - 11 illus - 1 table - Project No. 3A012501B813, Unclassified Report

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MEASUREMENT OF ADP:O VALUES IN RAT LIVER HOMOGENATES. II. EFFECTS OF PURINE AND PYRIMIDINE NUCLEOTIDES ON RESPIRATION AND PHOSPHORYLATION

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ABSTRACT

MEASUREMENT OF ADP:O VALUES IN RAT LIVER HOMOGENATES. II. EFFECTS OF PURINE AND PYRIMIDINE NUCLEOTIDES ON RESPIRATION AND PHOSPHORYLATION

OBJECT

To investigate further the significance of ADP:O values in rat liver homogenates.

RESULTS AND CONCLUSIONS

The effects of a number of nucleotides on respiration and phosphorylation were studied. In all cases where phosphorylation was stimulated, respiration increased correspondingly. Studies with C^{14}-labeled nucleotides gave no indication that non-phosphorylative side reactions interfere with ADP:O measurements. These findings provide additional evidence that ADP:O values are a reliable measure of oxidative phosphorylation in liver homogenates from normal rats.

RECOMMENDATIONS

None.

APPROVED: Walter F. Kocholaty
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APPROVED: Sven A. Bach
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I. INTRODUCTION

In the first paper of this series, a polarographic method for determining ADP:O[1] values in liver homogenates was described (1). The theoretical basis for ADP:O measurements requires that substrate-ATP respiration[2] responds specifically to small changes in the ADP concentration and that all exogenous ADP is phosphorylated. Ziegler, Strickland, and Anthony (1) showed that substrate-ATP respiration is altered by small changes in ADP concentration of liver homogenates. Under proper experimental conditions, exogenous ADP seems to be converted quantitatively to ATP (1). ADP:O values, therefore, appear to be a valid measure of oxidative phosphorylation in liver homogenates (1).

Nevertheless, in view of the numerous reactions that may occur in homogenates, additional studies were undertaken to search for side reactions that might alter the significance of ADP:O values. The present report describes the nucleotide specificity for stimulation of oxidative phosphorylation and the transformations of C^{14}-labeled adenine nucleotides in homogenates.

II. METHODS

Total liver homogenates were prepared in 225 mM mannitol, 75 mM sucrose, and 0.1 mM EDTA, pH 7.1, using first a Potter-Elvehjem and then a Dounce homogenizer (1). The reaction medium contained 15 mM sodium succinate, 45 mM mannitol, 15 mM sucrose, 0.02 mM

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[1] Abbreviations: M, moles per liter; AMP, ADP, ATP, CMP, CDP, CTP, UMP, UDP, UTP, IMP, IDP, ITP, GMP, GDP, GTP, 5'-mono-, di-, and triphosphates of adenosine, cytidine, uridine, inosine, and guanosine; cyclic AMP, adenosine-3', 5'-monophosphate; EDTA, sodium ethylenediaminetetraacetate; TCA, trichloroacetic acid; ADP:O value, ratio of moles of added ADP to gram atoms of the increase in oxygen consumed during the burst of respiration after ADP addition; ATPase activity, all reactions dephosphorylating ATP to ADP or AMP.

[2] The respiratory state is designated by listing, in order of addition, the reagents added to the system. The concentrations of reagents are given in parentheses where such information is pertinent.

EDTA, 40 mM KCl, 20 mM $MgCl_2$, and 20 mM potassium phosphate adjusted to pH 7.4, except where indicated otherwise. To obtain the maximal respiration, ADP (> 250 μM) was added within 1 or 2 minutes after the homogenate was treated with succinate. When addition of ADP was delayed 4 to 8 minutes, both ADP and ATP were required for maximal succinate oxidation. Respiration was measured polarographically in a sealed, 3.5 ml glass vessel, which was surrounded by a water jacket at 24°C (2, 3).

The phosphorylation of unlabeled nucleotides was followed by chromatography of perchloric acid extracts (1) on Dowex-1-Cl (200 mesh) 8X (4). Separation of various nucleotides was readily accomplished in most cases since each extract contained only a few nucleotides. The base moiety was identified from the ultraviolet absorption spectrum of the eluted compounds. The number of phosphate groups per nucleotide was determined by comparison of the elution sequence with that of authentic compounds (Fig. 1).

Paper chromatographic isolation of C^{14}-labeled compounds was carried out on lipid-rich and lipid-free extracts. One to 4 ml of homogenate which had been incubated with C^{14}-labeled nucleotides was added to 100 ml of boiling 80% ethanol (v/v) to inactivate the enzymes. The turbid solution was filtered through Whatman # 42 paper. The residue was washed once with 5 ml of water, twice with 5 ml of absolute ethanol, twice with 5 ml of methanol-toluene (3:1; v/v), twice with 5 ml of chloroform-methanol (2:1, v/v), and three times with 5 ml of chloroform. All washings were combined with the initial alcohol extract and concentrated to 5 ml. The concentrate was partitioned between 5 ml of water and 2 ml of chloroform. The water and chloroform phases were separated, concentrated to 0.2 ml, and chromatographed on Whatman #4 paper in phenol-water (100:38) and then butanol-propionic acid-water (142:71:100).

The residue was further washed with 5 ml of water and then 5 ml of 5% TCA at 0°C. These washings were discarded. The residue was then treated with 2 ml of 5% TCA at 100°C for 30 minutes. This nucleic acid-rich TCA extract was concentrated, dried on filter paper, and radioactivity determined with a Geiger-Müller tube.

ATP, ADP, and AMP were separated in the phenol-water and butanol-propionic acid-water solvent systems. In cases where both solvents were allowed to drip off the paper, AMP, IMP, and GMP could be separated. The radioactivity on paper chromatograms was located by autoradiography and measured with a Geiger-Müller tube.

ATP-8-C^{14} (specific activity, 1.1 μc/mg), uniformly labeled ADP-C^{14} (specific activity, 1.7 μc/mg), and AMP-8-C^{14} (specific activity, 3.1 μc/mg) were obtained from Schwarz BioResearch, Inc. Unlabeled nucleotides, except UDP and cyclic AMP, were from Sigma Chemical Company; UDP from Pabst Laboratories; cyclic AMP and sodium succinate, Grade A, from California Corporation for Biochemical Research; other reagents used in respiratory studies were Fisher Certified Reagents from Fisher Scientific Company. Preparation of reagents was the same as described earlier (1).

III. RESULTS

Effect of nucleoside diphosphates on respiration and phosphorylation. GDP, IDP, and UDP stimulated succinate respiration by less than 30% (Fig. 2); CDP was somewhat more effective, giving as much as 50% stimulation. In contrast, ADP increased succinate respiration by 200 to 400% (1). Chromatographic analyses of extracts of homogenates which had been incubated with succinate and GDP, CDP, or ADP for 5 minutes revealed that all of these were phosphorylated to some extent. ADP was phosphorylated three times faster than CDP and 19 times faster than GDP. Thus the ability of nucleoside diphosphates to stimulate respiration correlated with their ability to stimulate phosphorylation.

Prior treatment of the homogenate with ATP increased the response when non-adenosine diphosphates were added. Figure 3 shows that GDP, IDP, CDP, UDP, or ADP stimulated succinate-ATP respiration to the same extent. Furthermore, GDP, IDP, UDP, or CDP produced a burst of respiration, which was followed by a return to the initial succinate-ATP respiration (Fig. 4). A subsequent addition of nucleoside diphosphate produced a second burst of respiration. Similar results were obtained earlier with ADP (1). Chromatographic analyses confirmed that GDP, CDP, and ADP were phosphorylated during the accelerated phase of respiration. The decreased respiration after the burst indicates that the added nucleoside diphosphate had been converted to nucleoside triphosphate (see below).

No combination of nucleoside diphosphates produced a greater rate of succinate oxidation than did ADP alone. Addition of GDP (240 μM), CDP (350 μM), IDP (720 μM), or UDP (400μM) did not alter succinate-ADP (250-600μM) respiration.

Effect of nucleoside monophosphates on respiration and phosphorylation. CMP, UMP, GMP, IMP, or cyclic AMP had no

appreciable effect on succinate respiration (Fig. 5). AMP stimulated succinate respiration fourfold, although only after a 1- to 2-minute lag (Fig. 6). Low concentrations of AMP (100 μM) produced a burst of respiration after a brief lag. At high concentrations, AMP eventually stimulated succinate respiration to the same extent as ADP. Chromatographic analysis showed that AMP was phosphorylated to ADP and ATP during the phase of rapid respiration.

Several of the nucleoside monophosphates stimulated respiration of homogenates containing both ATP and succinate. CMP, UMP, or AMP immediately stimulated succinate-ATP respiration, whereas GMP, IMP, and cyclic AMP were ineffective (Fig. 7). When $MgCl_2$ was omitted from the reaction medium, IMP still did not stimulate succinate-ATP(1 mM) respiration; AMP remained effective. Succinate-ATP(1 mM)-AMP(> 100 μM) and succinate-ATP(1 mM)-UMP(600 μM) respiratory rates were as great as the maximal succinate-ADP rate. On the other hand, succinate-ATP(1 mM)-CMP respiration was never more than 75% of succinate-ADP respiration. At a CMP concentration of 280 or 560 μM, respiration was lower than when a concentration of only 120 μM CMP was present.

This finding suggested that high concentrations of CMP might inhibit oxidative phosphorylation. Direct tests, however, did not support this hypothesis. Succinate-ADP(420 μM) respiration was not decreased by 560 μM CMP. Prior treatment of homogenates with 560 μM CMP did not alter succinate-ADP(150 μM) respiration. The other nucleoside-5'-monophosphates, likewise, failed to inhibit succinate-ADP respiration.

Addition of low concentrations of CMP, UMP, or AMP gave a transient increase in succinate-ATP(1 mM) respiration, followed by a decrease to the initial succinate-ATP rate (Fig. 8). UMP and CMP were found by chromatographic analysis to be phosphorylated during the burst of respiration. Neither GMP nor IMP was phosphorylated. These findings indicate that the burst of respiration terminated when the added UMP, CMP, or AMP was phosphorylated to the corresponding nucleoside triphosphate (see below).

Effect of nucleoside triphosphates on homogenate respiration. CTP, UTP, GTP, or ITP had little or no effect on either succinate respiration (Fig. 9) or succinate-ATP(1 mM) respiration (Fig. 10). In contrast, ATP(1 mM) increased succinate respiration 105% (1).

Transformations of C^{14}-labeled adenine nucleotides. The results of studies on the transformations of AMP-8-C^{14}, ADP-C^{14}, and ATP-8-C^{14} are summarized in Table 1. In all experiments, the activity was recovered almost entirely in three areas on the paper chromatograms (Fig. 11). The Rf values for these areas corresponded to those of nucleoside triphosphate, nucleoside diphosphates, and AMP. Significant amounts of ITP, IDP, GTP, and GDP could not have been formed, because only the monophosphate form of adenine nucleotides can be converted directly to other purine nucleotides (5) and IMP and GMP were found not to be phosphorylated. Consequently, the compounds shown in Figure 11 were identified as ATP, ADP, and AMP.

A shift of C^{14}-label from AMP and ADP to ATP could only occur if the homogenates carried out phosphorylation reactions. The C^{14}-label could not be transferred so completely to ATP by an exchange reaction (6), because the initial amounts of AMP and ADP accounted for a large percentage of all nucleotides.

In all experiments, at least one chromatogram was loaded with sufficient activity to detect trace activity in compounds other than nucleoside triphosphates, nucleoside diphosphates, and AMP. The activities of all these other compounds are included in the last column of Table 1. In most cases, less than 1% of the activity was found in compounds other than ATP, ADP, or AMP. The lack of any significant formation of IMP or adenosine is noteworthy.

Some of the homogenate residues left after extraction of the water- and chloroform-soluble compounds were examined for possible activity in nucleic acids that were not previously extracted. The hot-TCA extract contained less than 0.2% of the total activity in the case of homogenates incubated with ADP-C^{14}. The other homogenate residues were not analyzed.

IV. DISCUSSION

In the present investigation adenine nucleotides were found to stimulate succinate respiration more effectively than uracil, guanine, hypoxanthine, or cytosine nucleotides. ADP was the only nucleotide which immediately stimulated succinate respiration many fold. After a lag of several minutes, AMP increased succinate respiration to the same level as did ADP. The effectiveness of AMP is expected because liver mitochondria have adenylate kinase activity (7). During the lag period after AMP addition, AMP would react with endogenous ATP to form ADP. Then ADP would stimulate oxidative phosphorylation.

The specificity of nucleotide required to stimulate succinate respiration in homogenates is the same as that reported for oxidative phosphorylation in submitochondrial particles (8). Furthermore, nucleotides stimulated respiration only when phosphorylation was stimulated. These findings confirm the conclusion drawn by Ziegler et al (1) that nucleotides increase homogenate respiration by stimulating phosphorylation.

When the homogenate contained exogenous ATP, several non-adenine nucleotides were able to stimulate respiration. IDP, CDP, UDP, or GDP stimulated succinate-ATP respiration as effectively as ADP. This appears to be due to nucleoside diphosphokinases (5), which permit transphosphorylation reactions between ATP and nucleoside diphosphates. The ability of CMP or UMP to stimulate succinate-ATP respiration indicates that the rate of transphosphorylation between ATP and CMP or UMP is sufficient to permit a rapid rate of oxidative phosphorylation. For ATP and IMP or GMP, the rate of transphosphorylation is insufficient to permit rapid oxidative phosphorylation.

The specificity of the nucleotide required for oxidative phosphorylation does not mean that homogenates cannot phosphorylate non-adenine nucleotide without exogenous ATP. In fact, liver homogenates phosphorylate many nucleotides (9, 10). Phosphorylation of non-adenine nucleotides, however, proceeds much more slowly than phosphorylation of ADP in dilute homogenates.

The high activity of many nucleoside mono- and diphosphokinases permitted indirect studies on the dephosphorylation of CTP, UTP, GTP, and ITP. The failure of these triphosphates to stimulate succinate-ATP (1 mM) respiration may indicate that these nucleotides are not dephosphorylated as rapidly as ATP. On the other hand, ATPase enzymes are nearly saturated at an ATP concentration of 1 mM. If the same enzymes also dephosphorylate CTP, UTP, GTP, and ITP as rapidly as ATP, non-adenine nucleoside triphosphates would not significantly stimulate succinate-ATP(1 mM) respiration.

The primary role of adenine nucleotides in oxidative phosphorylation necessitates additional consideration of their metabolism in connection with ADP:O measurements. Ziegler et al (1) reported that exogenous ADP was essentially all phosphorylated under proper experimental conditions. In the present study C^{14}-labeled nucleotides provided a sensitive method to look for possible side reactions, i.e., reactions that convert ADP to a compound that cannot be phosphorylated to ATP. These tracer experiments support the earlier finding (1) that

ADP is converted to ATP quantitatively during ADP:O measurements. Regardless of whether AMP-8-C^{14}, ADP-C^{14}, or ATP-8-C^{14} was added to the homogenates, most of the activity was recovered in ATP, a smaller part in ADP, and even less in AMP. Less than 2% of the activity was found in compounds other than adenine nucleotides. These findings provide additional evidence that ADP:O values are a valid measure of oxidative phosphorylation in liver homogenates from normal rats.

Calculated ADP:O values, however, do not necessarily measure oxidative phosphorylation under all conditions. In cases where the physiological state of rats is altered by experimental treatment, side reactions might remove exogenous ADP before it is all phosphorylated. Also possible side reactions of AMP must be considered, since some ADP is converted to AMP by adenylate kinase activity before being phosphorylated to ATP. For example, if adenylic acid deaminase activity were greatly increased in some abnormal condition, significant deamination of AMP to IMP could occur. Since IMP is not rapidly phosphorylated, the amount of nucleotide phosphorylated would be less than the amount added to the homogenate. In such a case, the calculated ADP:O value would be higher than normal. Also, in tissues such as brain and skeletal muscle, which may have higher adenylic acid deaminase activity than liver (11, 12), ADP:O values might not be a valid measure of oxidative phosphorylation.

If ADP:O values were found to change in homogenates from experimental animals, further studies would be needed to prove that the efficiency of oxidative phosphorylation has changed. In particular, one should demonstrate that no side reactions occur and that the ATPase correction (1) is still valid. Where these conditions are met, ADP:O values are a reliable measure of oxidative phosphorylation.

V. SUMMARY

ADP stimulated succinate respiration much more than UMP, GMP, CMP, IMP, cyclic AMP, UDP, GDP, CDP, IDP, UTP, GTP, ITP, or CTP. After a lag, AMP stimulated respiration as effectively as ADP. When homogenates were pretreated with ATP and succinate, CDP, UDP, GDP, IDP, CMP, or UMP immediately stimulated respiration; IMP, GMP, cyclic AMP, ITP, GTP, UTP, or CTP remained ineffective. In all cases where respiration was stimulated, phosphorylation increased correspondingly. Studies with AMP-8-C^{14}, ADP-C^{14}, and ATP-8-C^{14} gave no indication that non-phosphorylative side reactions interfered with ADP:O measurements. These findings

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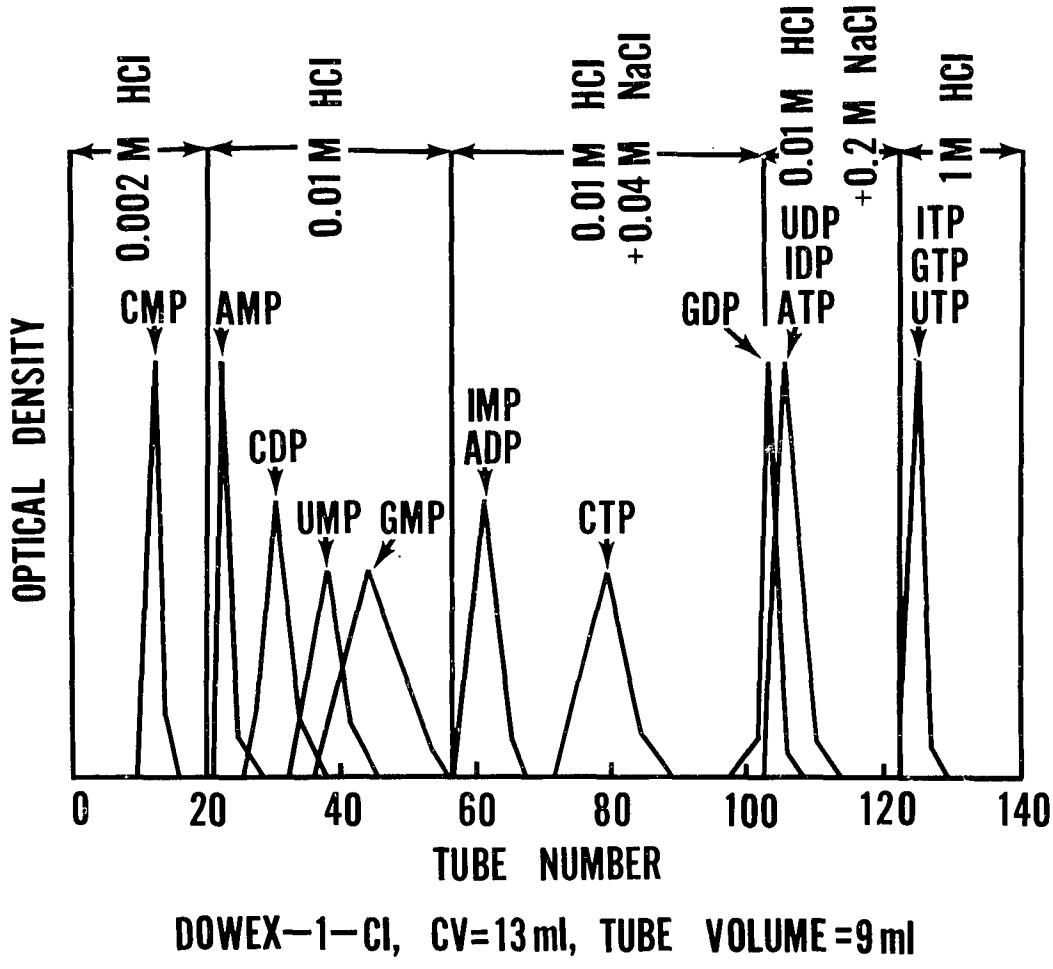

Fig. 1. Elution of nucleotides from Dowex-1-Cl. The optical density was measured at 260 mμ for adenine, guanine, uracil and hypoxanthine nucleotides and at 280 mμ for cytosine nucleotides. Column of resin was 20 cm x 0.63 cm^2; 9 ml fractions were collected.

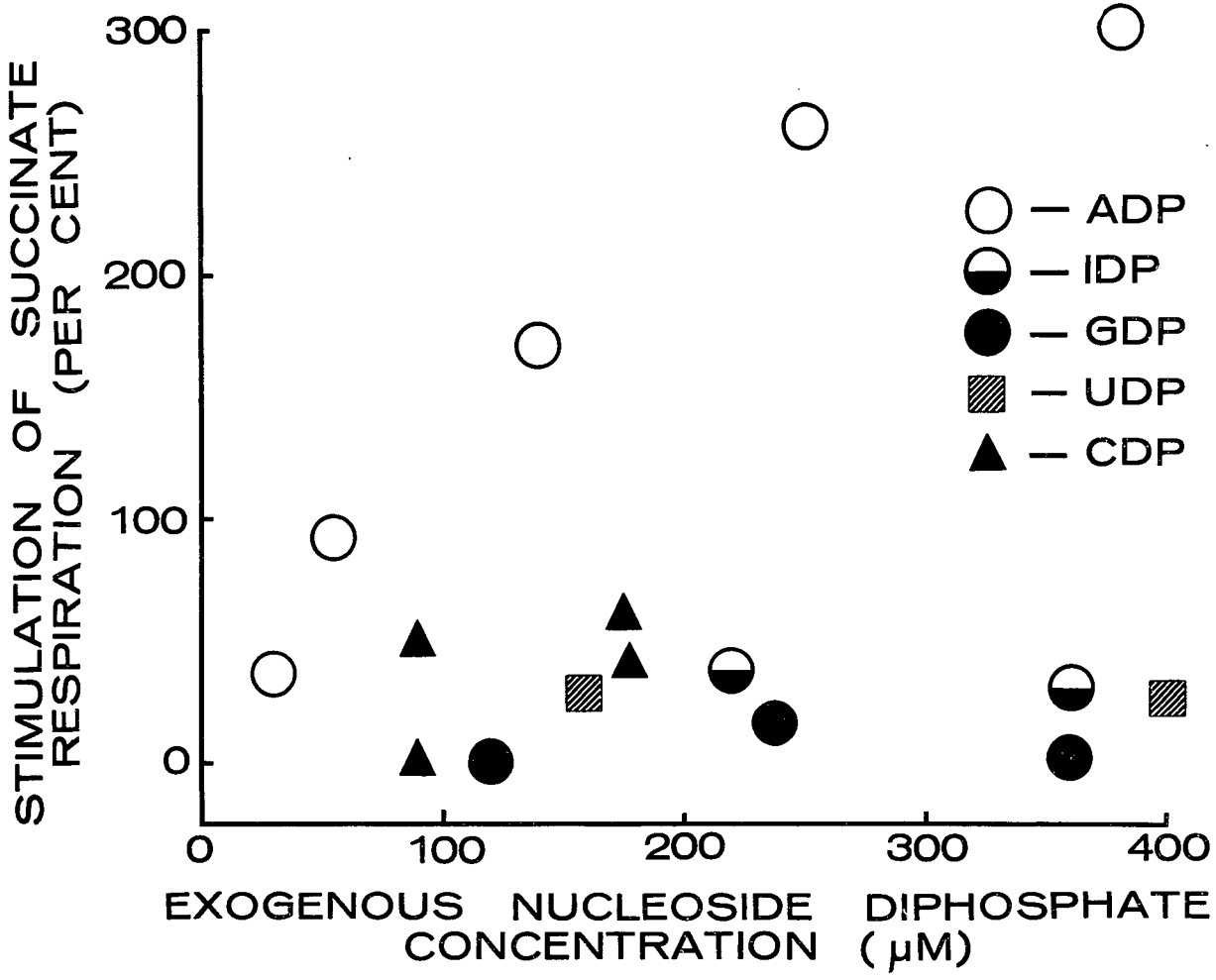

Fig. 2. Stimulation of succinate respiration by nucleoside diphosphates. Medium contained 5-9 mg homogenate protein.

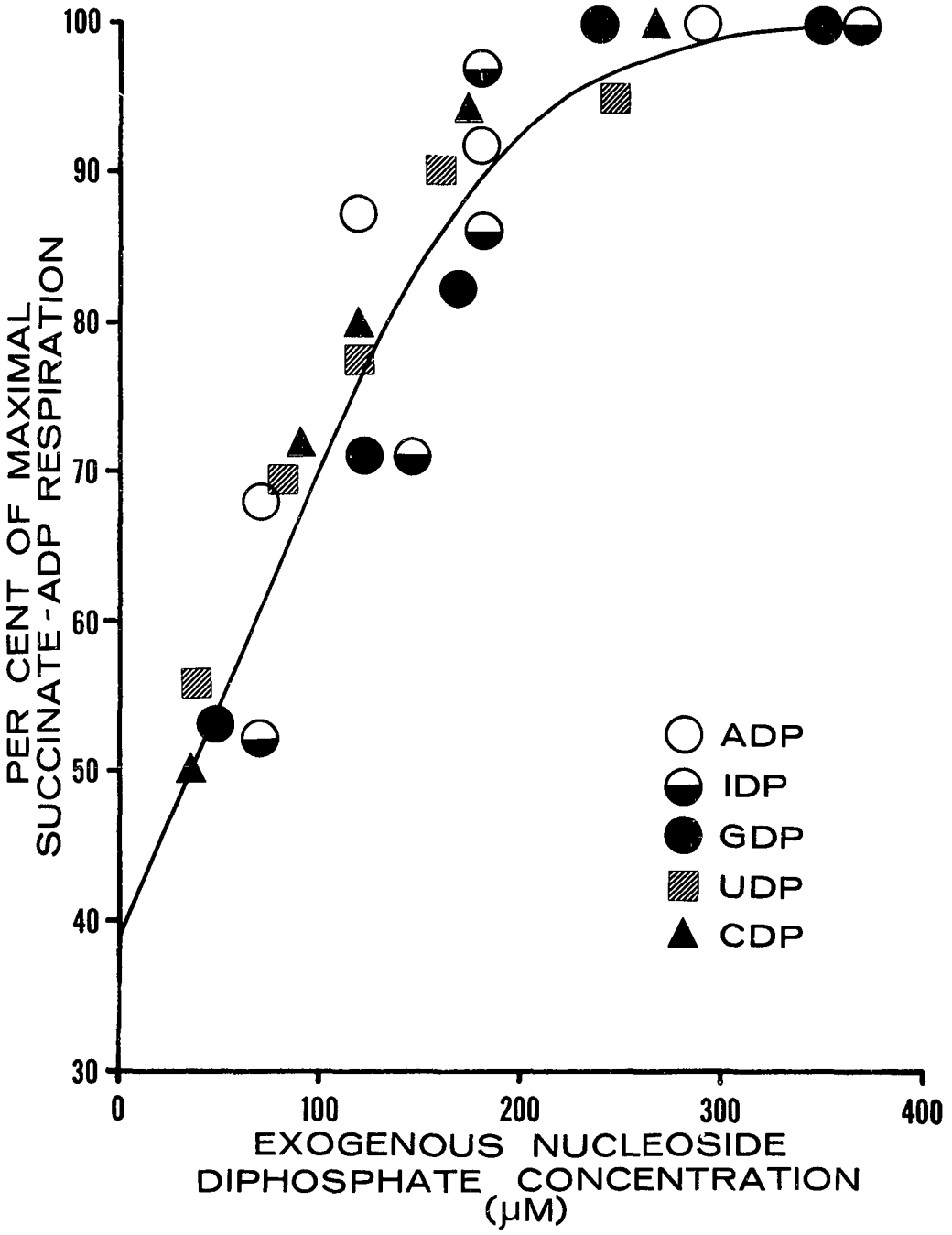

Fig. 3. Effect of nucleoside diphosphates on succinate-ATP respiration. Medium was supplemented with 1 mM ATP and contained about 3 mg protein.

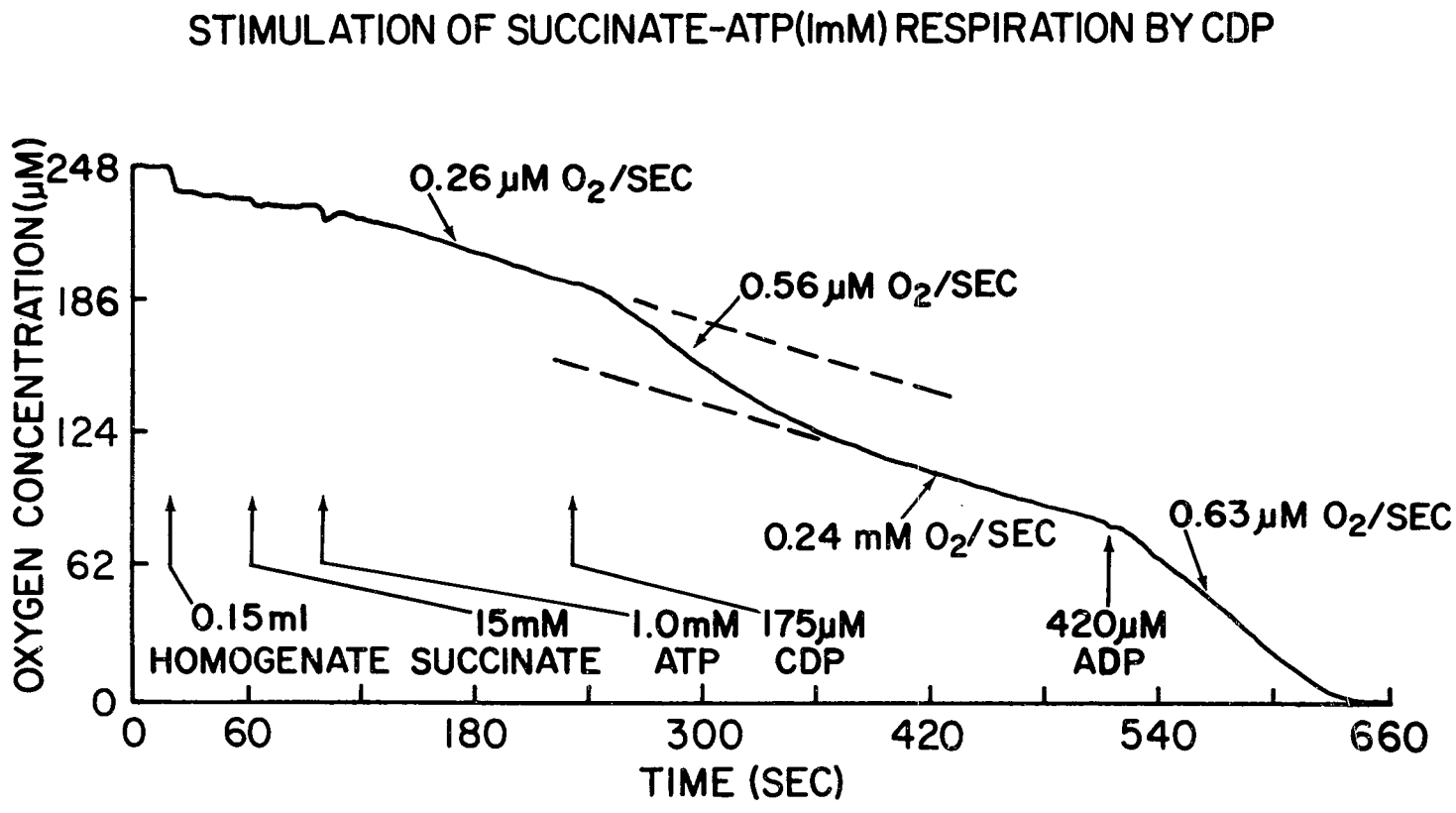

Fig. 4. Stimulation of succinate-ATP respiration by CDP. Medium did not contain succinate initially.

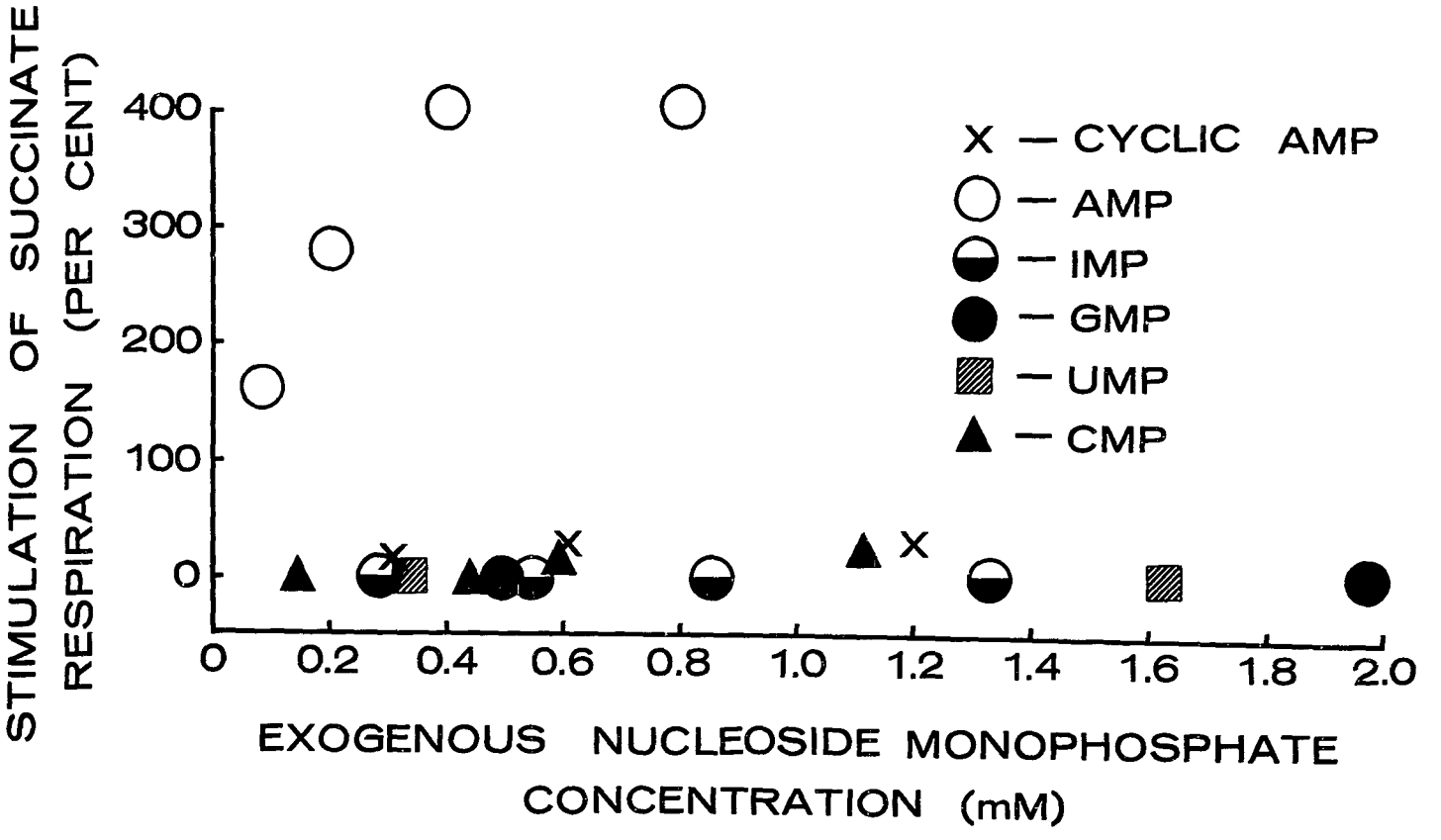

Fig. 5. Effect of nucleoside monophosphates on succinate respiration. Medium contained 5-6 mg protein.

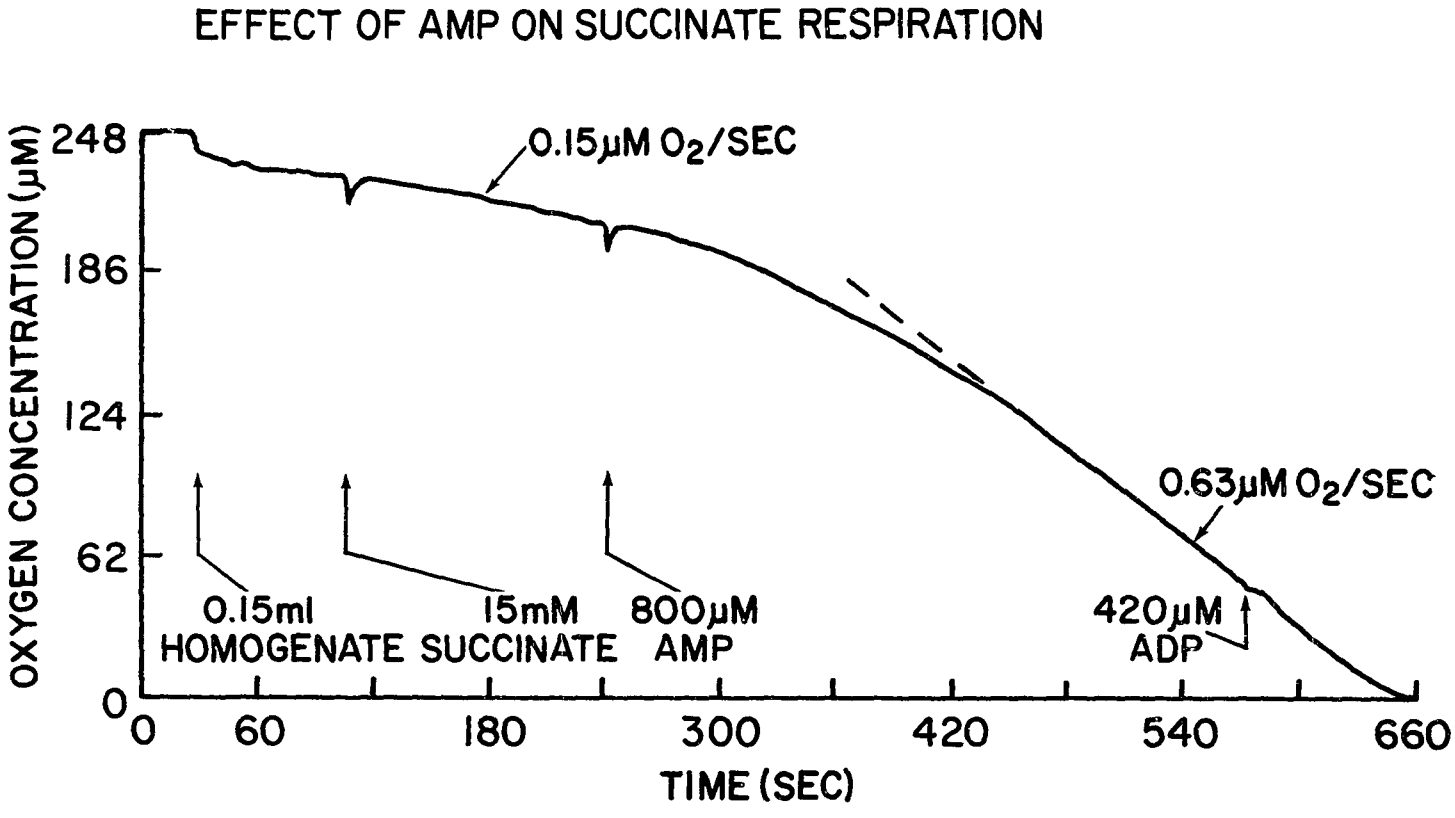

Fig. 6. Stimulation of succinate respiration by AMP. Medium was initially without succinate.

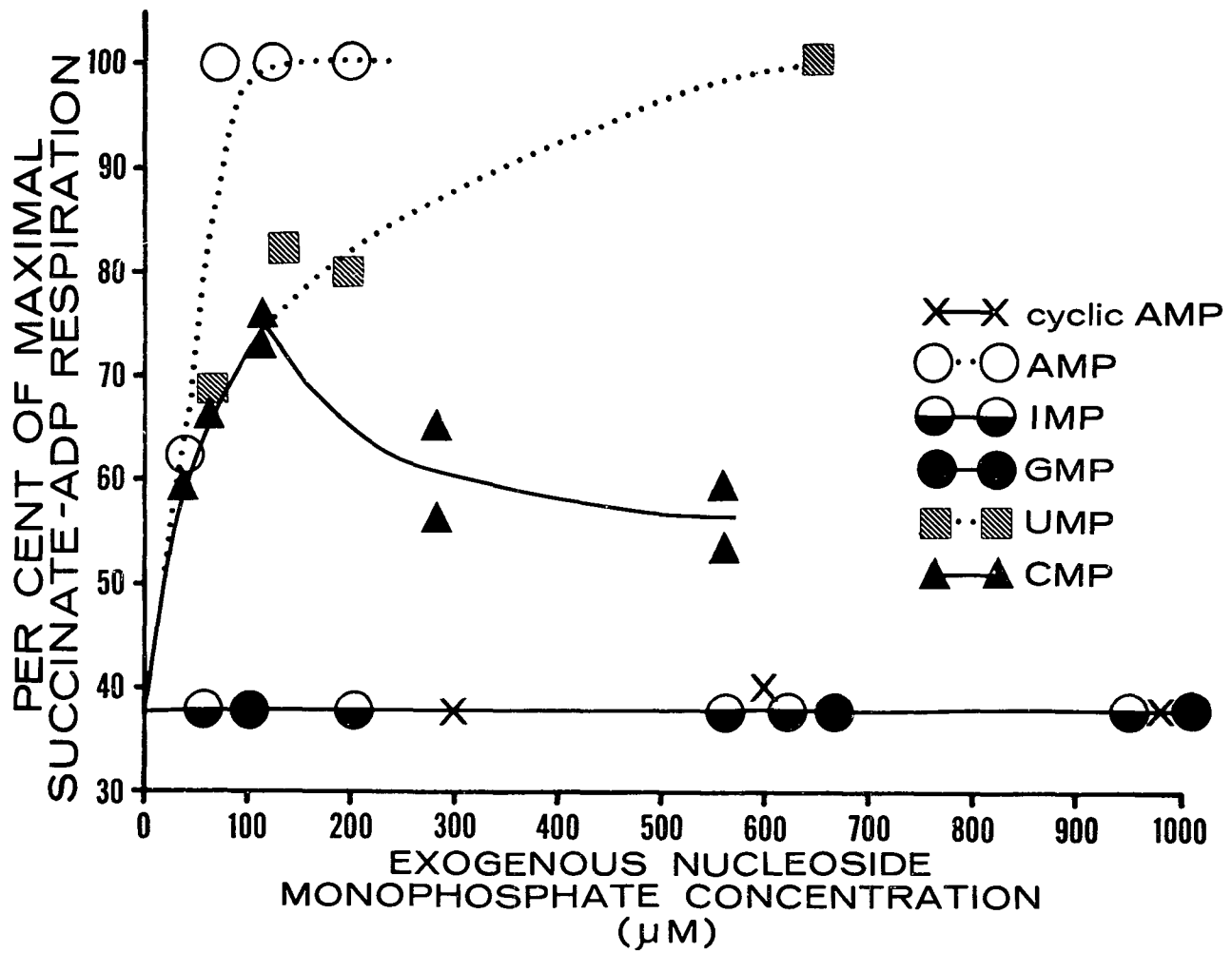

Fig. 7. Effect of nucleoside monophosphates on succinate-ATP respiration. Medium contained ATP (1 mM), and 3-5 mg protein.

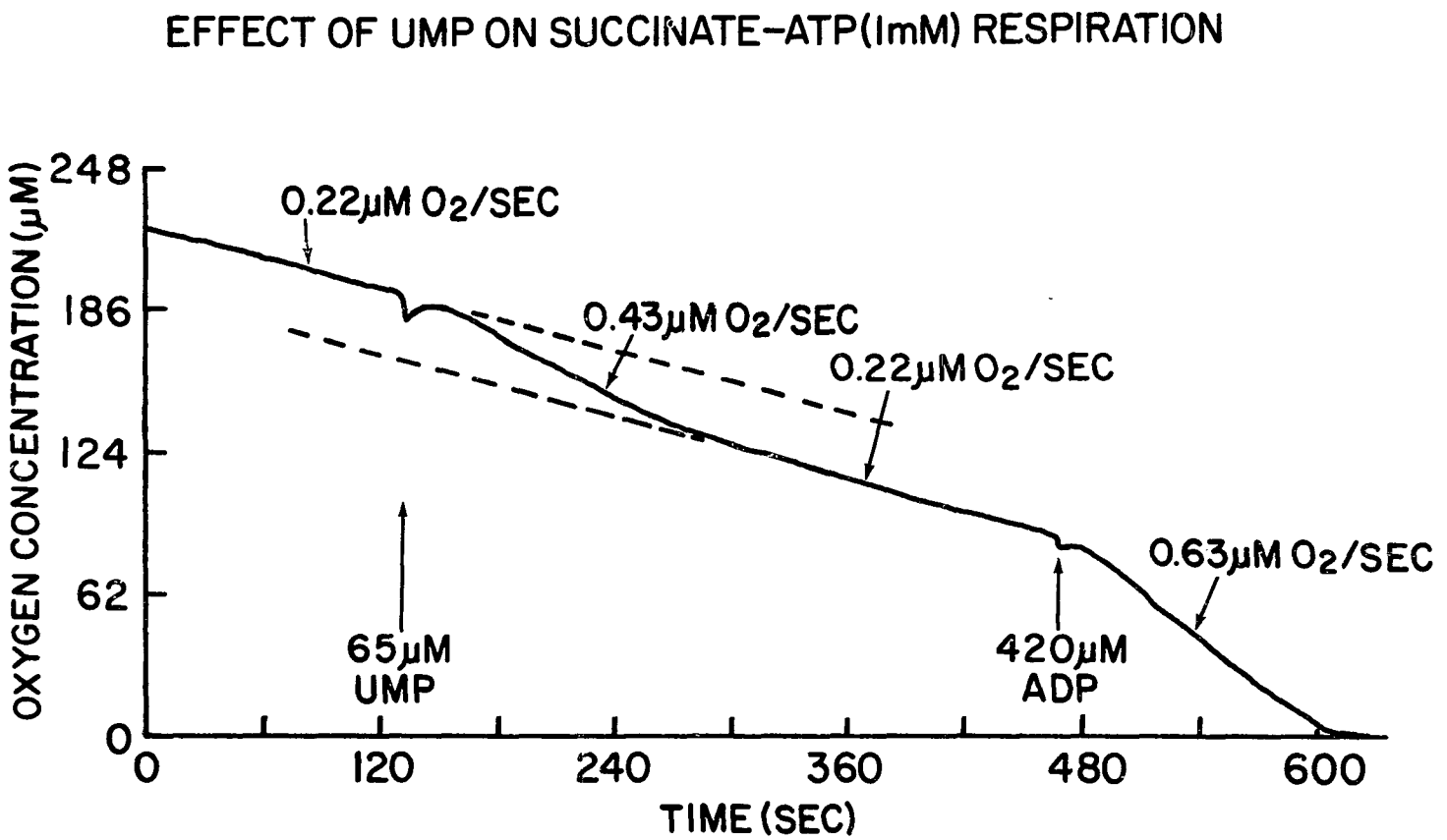

Fig. 8. Effect of UMP on succinate-ATP respiration. Medium contained ATP (1 mM).

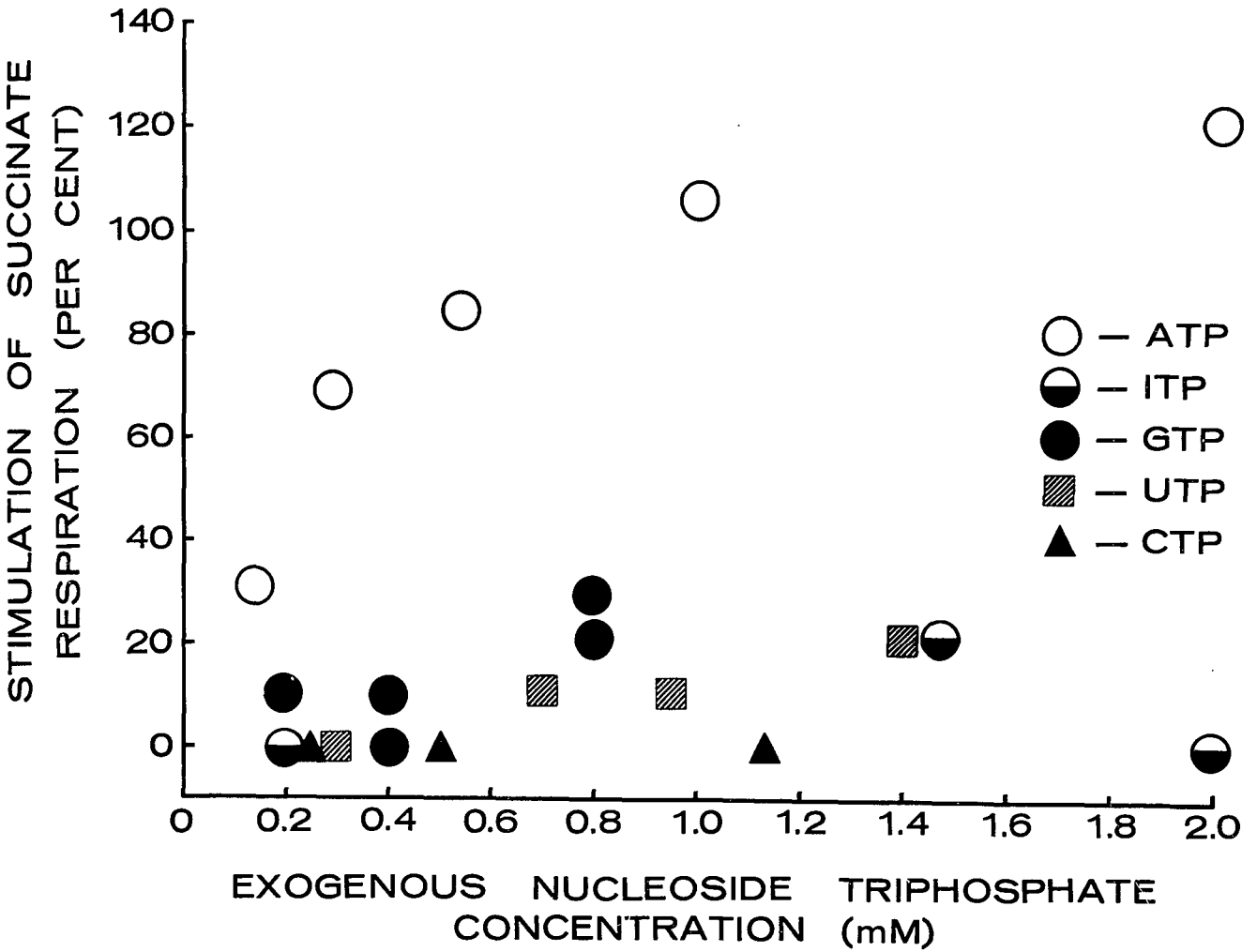

Fig. 9. Effect of nucleoside triphosphates on succinate respiration. Four-6 mg protein in medium.

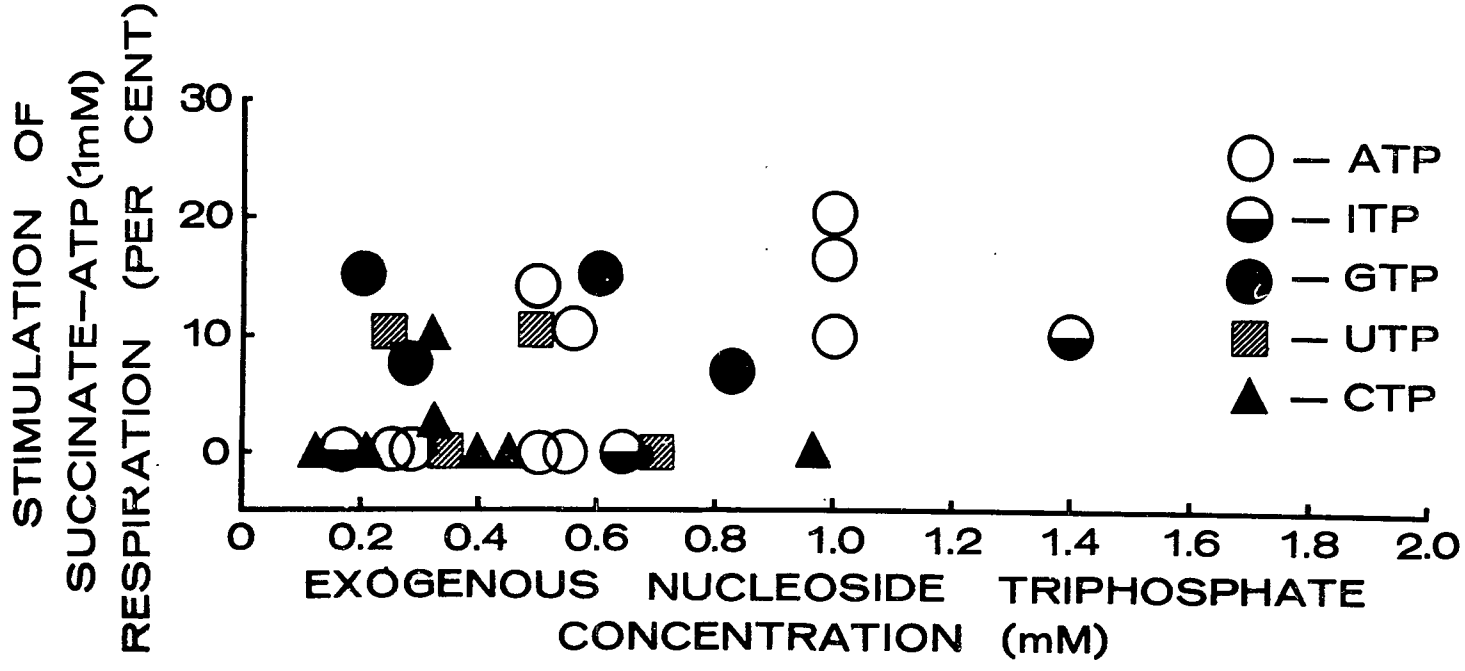

Fig. 10. Effect of nucleoside triphosphates on succinate-ATP(1 mM) respiration. Medium contained ATP (1 mM) and 3-5 mg protein. In the case of ATP, the abscissa indicates the concentration in excess of 1 mM.

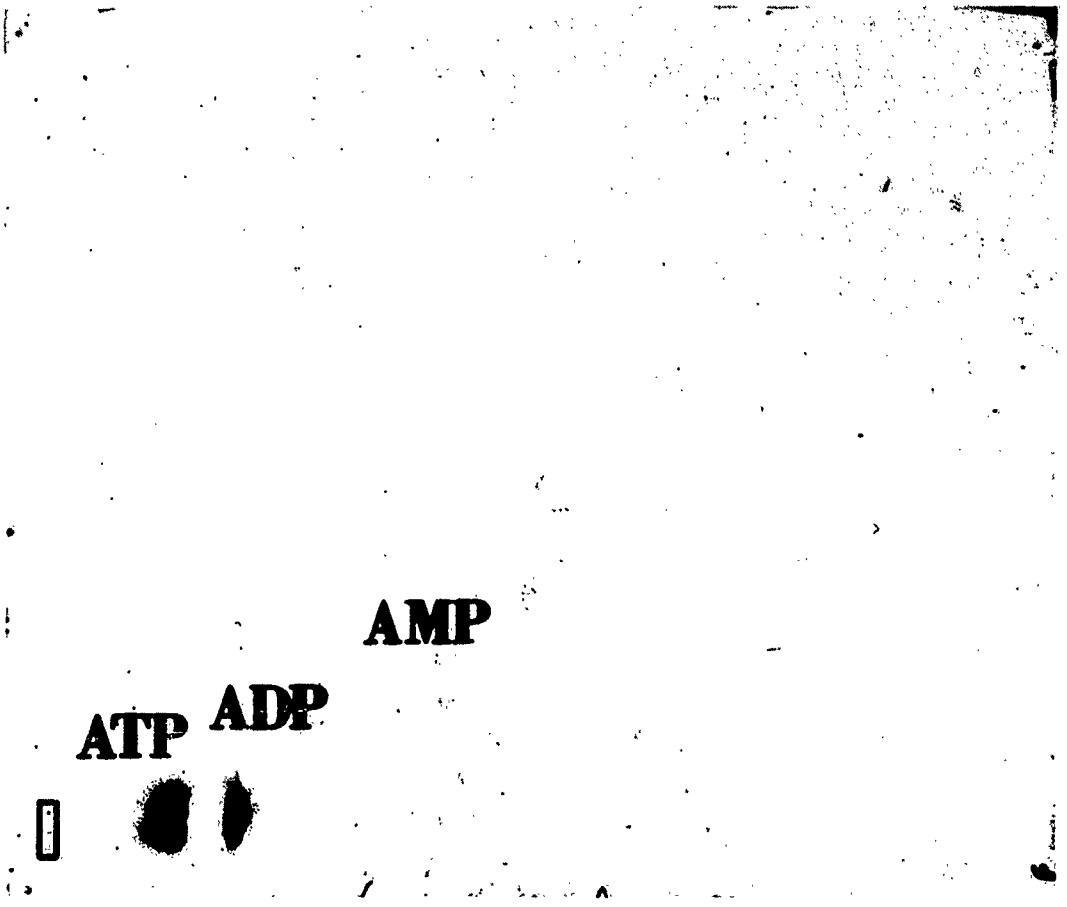

Fig. 11. Radiogram of water-soluble compounds from homogenate incubated with α-ketoglutarate and ADP-C^{14}. Incubation medium contained 45 mM mannitol, 15 mM sucrose, 0.02 mM EDTA, 40 mM KCl, 20 mM potassium phosphate, adjusted to pH 7.4. Chromatogram was developed in x-direction with phenol-water and in y-direction with butanol-propionic acid-water. Solvent fronts were at right and top edge of figure.

TABLE 1. TRANSFORMATIONS OF C^{14}-LABELED ADENINE NUCLEOTIDES

Incubations were carried out aerobically at 24°C. All reaction media contained 45 mM mannitol, 15 mM sucrose, 0.02 mM EDTA, 40 mM KCl, and 20 mM potassium phosphate, adjusted to pH 7.4. Other reagents added to the reaction medium are indicated in the table. AMP-8-C^{14} and ADP-C^{14} supplied by Schwarz BioResearch contained less than 1% of their activity in compounds other than AMP or ADP.

Initial Respiratory State	Incubation time, min.	mg homogenate protein per ml medium	Per Cent of Total Activity			
			ATP	ADP	AMP	Unidentified*
15 mM succinate, 20 mM $MgCl_2$, 1 mM ATP, 1 μc ATP-8-C^{14}	4	2.5	96	2.4	0.4	1.2
15 mM succinate, 20 mM $MgCl_2$, 0.4 mM ATP, 1.3 mM ADP, 1 μc ADP-C^{14}	4	3.2	84	14	0.8	1.6
15 mM α-ketoglutarate, 10 mM malonate, 0.8 mM ATP, 1.3 mM ADP, 1 μc ADP-C^{14}	4	6.0	68	27	5	< 0.3
15 mM succinate, 1 mM ATP, 0.1 mM AMP, 0.5 μc AMP-8-C^{14}	3	2.2	98+	+	0.5	< 1
15 mM succinate, 20 mM $MgCl_2$, 1 mM ATP, 0.1 mM AMP, 0.5 μc AMP-8-C^{14}	3	2.2	77	22	0.4	< 0.4
15 mM succinate, 0.1 mM AMP, 0.5 μc AMP-8-C^{14}	6	2.2	96	3.2	0.6	0.5

*Low activity prevented identification of these compounds, which were located on chromatograms in area where adenosine and adenine are found. No activity was recovered in compounds with high solubility in chloroform.

+Value includes ATP and ADP.